CHARACTERIZATION OF THE STK15 ONCOGENE IN BREAST TUMORS

Carrie A. B. Blanchette and Jeffrey R. Marks

Duke University Medical Center, Durham, NC 27710

cab13@duke.edu

Chromosomal instability, leading to aneuploidy, is a hallmark of nearly all solid cancers. One proposed route to chromosomal instability is via defective centrosome function. In several cancers, centrosomes appear abnormal in number, size, and position in the cell. Amplified centrosomes may occasionally form multipolar spindles, segregating chromosomes unequally to daughter cells and resulting in a state of aneuploidy. Although most of these aneuploid cells will die, a rare daughter cell may have the right chromosome complement to confer survival and even a growth advantage over neighboring normal cells, thus beginning the process of tumor progression. Therefore, genes involved in centrosome function may be important in the etiology of breast cancer. One such gene, STK15 (BTAK/aurora A), has been implicated in centrosome amplification. Previous studies have demonstrated that centrosome amplification, aneuploidy, and cellular transformation occur when STK15 is ectopically overexpressed in Rat1 fibroblasts and NIH3T3 cells in vitro, suggesting that STK15 is an oncogene. The STK15 gene is amplified in about 12% of primary breast tumors and is overexpressed at the mRNA level in a majority of primary breast tumors. However, the temporal relationship between aneuploidy and STK15 gene amplification / mRNA overexpression is still unclear.

Our goal was to obtain a complete measure of STK15 gene amplification, mRNA overexpression, and protein overexpression in a panel of approximately 50 primary breast tumors. We have developed real-time quantitative PCR assays to quickly and accurately measure STK15 gene amplification and mRNA overexpression using limited quantities of starting material. Preliminary data shows STK15 mRNA overexpression in the majority of the tumors we have examined, consistent with other published data. We have examined STK15 protein levels with an immunoblot assay. Finally, we have correlated this data to various clinical measures, such as estrogen receptor status, p53 genotype status, and most importantly, ploidy. These results may shed some light on the relationship between STK15 amplification / overexpression and aneuploidy in breast cancer. These studies will increase the general understanding of the role of STK15 in breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0229 supported this work.

C-MYC-INDUCED MAMMARY TUMORIGENESIS SELECTS FOR SPONTANEOUS ACTIVATING RAS MUTATIONS

R. B. Boxer, C. M. D'Cruz, E. J. Gunther, J. L. Hartman, L. P. Sintasath, J. D. Cox, S. E. Moody, S. I. Ha, G. K. Belka, and L. A. Chodosh

University of Pennsylvania School of Medicine

rboxer@mail.med.upenn.edu

Among the different genetic lesions implicated in human breast cancer, amplification of the *c-MYC* oncogene has been found in five to twenty percent of breast tumors. Our laboratory has developed a bitransgenic model in which the tetracycline regulatory system is used to conditionally express the *c-MYC* oncogene in the mammary epithelium of mice. We have used this model to demonstrate that approximately half of c-MYC initiated mammary adenocarcinomas remain dependent on c-MYC expression for maintenance of the tumorigenic state, while other c-MYC initiated tumors acquire the ability to grow in the absence of c-MYC overexpression. In addition, we have found that approximately fifty percent of c-MYC initiated mammary adenocarcinomas harbor spontaneous activating point mutations in *ras* gene family members, with a highly significant preference for these mutations to occur in *K-ras*. Mutations in *K-ras* were found nearly three times more frequently than mutations in *N-ras* and no mutations were detected in *H-ras*. Interestingly, the presence of an activating mutation in *K-ras* was found to correlate strongly with the failure of tumors to regress fully following *c-MYC* deinduction in our bitransgenic model. Nearly all tumors with detectable activating *K-ras* mutations exhibited only partial regression, whereas the majority of tumors without detectable activating *ras* mutations fully regressed. Surprisingly, following *c-MYC* transgene deinduction, tumors with detectable activating *N-ras* mutations behaved more similarly to tumors without a detectable *ras* mutation than to tumors with activating *K-ras* mutations. When analyzed for Ras activity, *K-ras* mutant tumors were found to have higher levels of Ras-GTP than tumors that did not have detectable *ras* mutations and that fully regressed following *c-MYC* transgene deinduction. Furthermore, *N-ras* mutant tumors demonstrated intermediate levels of Ras-GTP, whereas incompletely regressing tumors without detectable *ras* mutations displayed Ras-GTP levels comparable to those of *K-ras* mutant tumors. These data suggest that spontaneous activating mutations in both *K-ras* and *N-ras* represent a preferred secondary pathway for c-MYC induced tumorigenesis in the mammary gland. However, despite the high degree of homology between these genes, K-ras activation has consequences that are distinct from those conferred by N-ras activation. This is suggested both by our observation that *K-ras* mutation is more frequent than *N-ras* mutation in c-MYC induced mammary adenocarcinomas and by our finding that K-ras activation appears to confer c-MYC independent growth, whereas N-ras activation does not.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0397 supported this work.

PI3K/AKT2 PATHWAY MEDIATES HER-2/NEU-INDUCED TELOMERASE ACTIVATION IN HUMAN BREAST CANCER CELLS

AuHua Yang, June E. Paciga, Domenico Coppola, Sue A. Shelley, Santo V. Nicosia, and Jin Q. Cheng

Department of Pathology and Programs of Molecular Oncology and Drug Discovery, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, FL 33612

jcheng@hsc.usf.edu

Telomerase maintenance has been proposed as an essential prerequisite to human tumor development. However, the molecular mechanisms that activate this enzyme during neoplastic transformation are not well understood. Previous studies demonstrated frequent overexpression of Her2/neu and activation of AKT2 in human breast carcinoma. Here, we show that Her2/neu induces telomerase activity in human mammary epithelial cells. Her2-stimulated telomerase activity was inhibited by PI3K inhibitors, wortmannin and LY294002, dominant negative AKT2 and PTEN tumor suppressor. Expression of constitutively active PI3K (p110*) and AKT2 induced telomerase activity. The levels of human telomerase reverse transcriptase (hTERT) mRNA were increased after introduction of Her2, p110* or constitutively active AKT2 into MCF-10A cells. Even though hTERT contains three Akt phosphorylation consensus sequences, hTERT was not phosphorylated by constitutively active AKT2 as demonstrated by in vivo [32P]-orthophosphate labeling. Moreover, hTERT-3A, by converting 3 potential Akt phosphorylation sites to alanine, had the same level of the activity as that of wild type hTERT. In addition, hTERT promoter activity was significantly induced by ectopic expression of Her2, p110*, and constitutively active AKT2. We have also demonstrated overexpression of AKT2 protein in 38 of 69 primary breast carcinomas. The majority of the cases with elevated levels of AKT2 are Her2/neu and hTERT positive. These findings suggest that regulation of telomerase by Her2-PI3K-AKT2 at transcriptional level could play a pivotal role in breast cancer development.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0394 supported this work.

CHARACTERIZATION OF THE 17Q23 AMPLICON

Sinclair, C.S., Zang, Y., and Couch, F.J.

Department of Laboratory Medicine and Pathology,
Mayo Clinic

couch.fergus@mayo.edu

Comparative genomic hybridization (CGH) of breast tumors and cancer cell lines has identified several amplicons in breast tumors including the 17q22-23 region. Amplification of the 17q22-23 region has been detected in 18% to 31% of primary breast tumors, 41% of breast tumor metastases, 50% of tumors containing BRCA1 mutations, 87% of breast tumors containing BRCA2 mutations, and in aneuploid tumors. To characterize the structure of the amplicon we initially generated a complete physical map of the 4 Mb region using 33 overlapping BAC clones. Detailed copy number analysis of 85 EST and STS probes from the region in breast cancer cell lines and tumors was carried out by Southern blotting. In addition the 33 BAC clones from the physical map were used for FISH analysis of the same cell lines and tumors. Seven specific and independent amplification maxima were identified. A transcription map was generated using known genes, ESTs, and predicted genes from the region. A total of 42 genes were mapped to the 4 Mb amplicon including 10 genes that map at the amplification peaks. Expression analysis has confirmed that the 10 amplified genes are also overexpressed in breast tumors and cell lines. Several of these genes have been evaluated as candidate oncogenes and two appear to contribute to breast cancer. TBX2 is a transcription factor that enhances cell proliferation by inhibiting senescence, while RPSK6B1 (p70-S6-kinase) regulates early response gene translation and the G1/S cell cycle checkpoint, and also correlates with poor breast cancer patient survival.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9282 supported this work.

IN VIVO CLONING OF A NEW GROWTH SUPPRESSOR GENE AS A DIRECT TARGET OF EGR-1

Ian de Belle, Jie-Xin Wu, Sabina Sperandio, and Eileen D. Adamson

La Jolla Cancer Research Center,
The Burnham Institute, La Jolla, CA 92037

idebelle@burnham.org

Egr-1 is an immediate early gene transcription factor, responding to diverse stimuli and affecting downstream target gene transcription to accomplish its biological effects. One important effect of Egr-1 expression is to decrease the growth and tumorigenic potential of several tumor cell types. The purpose of this study is to identify the Egr-1 target gene(s) important for its function. In order to better understand the activity of a transcription factor it is desirable to identify the complement of true, in vivo targets for that factor in a given cell type under a defined experimental condition. To address this issue we have elaborated a methodology involving formaldehyde-induced in vivo protein/DNA crosslinking, chromatin immunoprecipitation and multiplex PCR. For the first time using this approach, we report the cloning of a new Egr-1 target gene which is able to account for, at least in part, the growth inhibitory activity of Egr-1. We have named this new protein TOE1 for Target Of Egr-1. Over-expression of intact but not mutant TOE1 produces high levels of the cell cycle inhibitor, p21WAF1, thus accounting for the suppressed growth of TOE1-expressing cells. TOE1 is similarly induced in breast cancer cells exposed to Egr-1 stimulating factors such as TPA. Another component in the mechanism of action of TOE is its ability to complex with p53 which further modulates p21 expression and represses growth. Using this methodology, we have demonstrated that in vivo physiologically relevant gene targets can be identified, and, in so doing, provide a clearer picture of transcription factor activities that function differentially in normal and breast cancer cells.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9092 supported this work.

IDENTIFICATION OF ESX TARGET GENES

Jason D. Prescott and Arthur Gutierrez-Hartmann[1]

[1]University of Colorado,
Comprehensive Cancer Center, Denver, CO

jason.prescott@uchsc.edu

ESX is a novel member of the proto-oncogenic Ets factor family, a diverse group of transcription factors involved in differentiation, cell cycle control, and development. ESX expression is restricted to epithelial cells and has been implicated in human breast carcinogenesis. ESX over-expression is detected in 40% of human breast ductal carcinomas in situ. Further, we have shown that stable expression of recombinant ESX protein imparts a transformed phenotype to the normally nonmalignant, ESX-negative MCF12A human breast cell line. Additionally, inhibition of endogenous ESX expression in the T47D human breast cancer cell line blocks the transformed phenotype in these cells. Yet, while these observations strongly suggest a role for inappropriate ESX action in breast cancer, relatively little is known about the specific mechanism(s) whereby ESX brings about carcinogenesis. Using transient expression approaches, we have demonstrated that GFP-ESX fusion protein and HA-tagged ESX are localized to the nucleus of transfected MCF12A cells. Further, we have used DNA micro-array analysis to identify putative ESX target genes and then used the Chromatin Immunoprecipitation (ChIP) assay to validate that such genes are indeed primary targets. Having optimized the ChIP assay, we also show the in vivo interaction between ESX protein and the genomic HER-2 promoter in MCF12A cells. Subsequently, we have shown up-regulation of HER-2 protein expression in ESX expressing MCF12A cells, demonstrating the functional significance of binding between ESX and the HER-2 gene. Taken together, these studies show that ESX is a regulator of the several target genes in vivo and suggest that pharmacological targeting of ESX may inhibit the particularly aggressive breast cancer phenotype associated with HER-2 gene expression.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0474 supported this work.

DISSECTING THE GENETIC BASIS AND THE ROLE OF ABERRANT SIGNALING IN BREAST CANCER DEVELOPMENT

James J. Fiordalisi and Channing J. Der

University of North Carolina at Chapel Hill

fiordali@radonc.unc.edu

The complex nature of the genetic events that trigger the development of breast cancer remain to be identified. Our laboratory has used two approaches to define these events. First, we have developed and refined a retrovirus-based gene transfer assay to identify activated oncogenes in breast cancer. This approach involves the use of a functional screen to identify genes expressed in breast cancer cells that exhibit the ability to promote the uncontrolled growth of normal cells. We have generated several retrovirus-based cDNA expression libraries that represent genes expressed in noninvasive (MCF-7 and T47D) and invasive (MDA-MB4682 and BT549) human breast cancer cell lines. These libraries have been introduced into Rat-1 rodent fibroblasts and RIE-1 rat intestinal epithelial cells and transforming activity was then assayed for. Two isolates from our analyses encode the Raf-1 serine/threonine kinase and the fibroblast growth factor receptor 2. Second, we have evaluated the role of aberrant signal transduction in promoting the aberrant growth of breast cancer cells. These studies are based on observations from model fibroblast cell systems, where it has been shown that the transforming activity of the HER2/ErbB2 receptor tyrosine kinase is dependent on the activation of the Ras oncoprotein. Additionally, the transforming activity of Ras has been shown to be dependent on activation of the ERK mitogen-activated protein kinase cascade as well as the phosphatidyl inositol 3-kinase (PI3K)/Akt serine/threonine kinase cascade. However, whether aberrant activation of HER2 in breast cancers actually leads to the activation of Ras and these two signaling pathways has not been determined. Therefore, we evaluated the activation of HER family receptors (1-4), Ras, ERK, and Akt in a panel of human breast carcinoma cell lines. We found that Ras activation strongly correlated with HER2 activation, but that ERK and Akt activation did not strongly correlate with Ras activation. Finally, we determined that neither ERK or Akt activation alone was necessary for breast cancer cells to overcome matrix-deprivation induced apoptosis, or anoikis. Taken together, our studies emphasize the complex and important role of aberrant signaling in breast cancer development.

The U.S. Army Medical Research Materiel Command under DAMD17-99-1-9331 supported this work.

THERAPEUTIC TARGETS DOWNSTREAM OF AKT KINASES

Thomas F. Franke,[1] Grigoriy A. Shostak,[1] Karen B. Weinshelbaum,[1] Sarah F. Giardina,[1] and Amy D. Tiersten[2]

Departments of [1]Pharmacology and [2]Medicine, Columbia University, 630 West 168th Street, New York, NY 10032

tff5@columbia.edu

The pathological activity of signal transduction pathways leading to activation of the serine-threonine kinase AKT (also called protein kinase B=PKB) is associated with tumor cell resistance against clinical antitumor treatment such as chemotherapy and irradiation.

To define the molecular targets downstream of AKT kinases that could lead to novel therapeutic strategies in mammary cancer disease, we have examined the regulation of various protein substrates of AKT including apoptosis signal regulating kinase (ASK1). During these studies, we found that intact ASK1 expression in cells was required for the efficient induction of cell death after exposure to chemotherapeutic drugs including taxanes. In addition, phosphorylation of ASK1 by AKT on serine residue-83 resulted in decreased ASK1 activation following various apoptotic insults such as oxidative stress and survival factor withdrawal, and decreased ASK1 activity was indicative of reduced apoptosis induction. To study the regulation of ASK1 by AKT, we generated phosphorylation-specific antibodies that only recognized ASK1 kinase phosphorylated in the AKT phosphorylation motif. By testing established tumor cell lines and also by examining human tumor specimen from patients with advanced breast cancer disease, we have found that increased ASK1 phosphorylation by AKT correlated positively with increased apoptosis resistance. The importance of ASK1 as a molecular target of AKT in pathological apoptosis regulation was further supported by experiments that have used mutant ASK1(S83A) to restore chemotherapeutic sensitivity in cancer cells overexpressing activated AKT. Taken together, we concluded that ASK1 is an important downstream target of AKT in pathological apoptosis regulation where selective restauration of ASK1 function could become an important aspect of tumor-specific therapeutic strategies.

Our findings of molecular mechanisms of AKT-dependent apoptosis suppression are relevant to researchers and the general public.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0214 supported this work.

DETERMINANTS OF AKT-DEPENDENT CHEMORESISTANCE

Sarah F. Giardina, Grigoriy A. Shostak, Karen B. Weinshelbaum, and Thomas F. Franke

Department of Pharmacology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032

tff5@columbia.edu

A frequent consequence of pathological changes in signal transduction pathways leading to activation of the serine-threonine kinase AKT (also called protein kinase B=PKB) is the acquisition of apoptosis resistance against chemotherapeutic drugs including the topoisomerase II inhibitor etoposide (Songyang et al., PNAS 94:11345-11350).

In our current study, we examined the molecular mechanisms underlying the chemoresistance of human cancer cells against etoposide and other anti-cancer drugs. Using pharmacological inhibitors and following adenoviral gene transfer of dominant-negative and activated signaling mutants of AKT, we found that inhibition of AKT activity in cancer cells rapidly and efficiently sensitizes cancer cells to drug-induced apoptosis as measured by characteristic morphological changes, activation of caspases and DNA fragmentation. To identify the molecular targets of AKT-dependent chemoresistance, we first examined the release of mitochondrial apoptotic complementation factors after etoposide treatment and following AKT inhibition. By using cell fractionation techniques and immunofluorescence analysis, we observed that etoposide treatment alone induced mitochondrial damage independently of AKT inhibition as measured by the release of mitochondrial cytochrome c. Depending on the cell type under investigation, however, subsequent activation of caspase-3/7 and apoptosis induction did not occur unless the AKT pathway was also inhibited. Our data suggested that AKT inhibition was required to facilitate the subsequent activation of caspase-3-like activities in cell lysates by cytochrome c or other mitochondrial complementation factors. Thus, we examined whether the caspase-9-dependent activation of caspase-3 downstream of mitochondrial damage was inhibited by pathologically increased AKT activity. By using structure-function analysis and in vitro reconstitution experiments using mutant caspase-9, we determined that direct inhibition of caspase-9 by AKT played only a partial role in inhibiting downstream caspases and that additional factors requiring ongoing macromolecular synthesis were necessary for optimal caspase activation.

Our findings of molecular mechanisms of AKT-dependent apoptosis suppression are relevant to researchers and the general public.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9153 supported this work.

OVEREXPRESSION OF UPSTREAM STIMULATORY FACTOR 2 (USF-2) IN THE MAMMARY GLAND REDUCES THE GROWTH OF MYC-DEPENDENT TUMORS

Hadsell, D.L., George, J., Torres, D.T., and Klementidis

Department of Pediatrics, USDA Children's Nutrition
Research Center, Baylor College of Medicine,
Houston, TX 77030

dhadsell@bcm.tmc.edu

USF-2 is a bHLH/zip protein, which shares DNA-binding specificity and structural characteristics with c-myc. Myc is known to stimulate the proliferation of mammary cells and is frequently amplified or overexpressed in breast cancer. Overexpression of USF-2 has been demonstrated to antagonize myc–dependent proliferation of transformed cells in culture. In some cell culture models, however, USF has been demonstrated to supplant myc in the regulation of specific genes The hypothesis tested in these studies is that overexpression of USF in the mammary glands of transgenic mice will inhibit myc-dependent tumorigenesis.

Overexpression of USF-2 in the mammary glands of transgenic mice was accomplished by constructing a transgene which encoded for a FLAG-tagged form or USF-2 under the control of the mouse mammary tumor virus (mmtv) long terminal repeat. Of eight lines of transgenic mice that were generated, one demonstrated expression of flag-tagged USF-2 in the lactating mammary gland at levels 12-fold over that of endogenous USF-2. Immunoflourescent staining of mammary tissue from lactating transgenic mice demonstrated intense nuclear localization for USF-2. Analysis of mammary gland development and lactation in these mmtv-USF-2 transgenic mice demonstrated that overexpression of USF-2 had little impact on lactational capacity, mammary gland development or the abundance of milk proteins. Evaluation of tumorigenesis in these mice (n=13) out to 316 days of age suggests that USF-2 when overexpressed by itself is not oncogenic. In contrast, 78% of mmtv-myc mice (n=9) analyzed have developed mammary tumors with an average latency of 146±13 days. Tumor frequency and latency in bigenic mmtv-USF-2/mmtv-C-myc mice out to 150 days of age was similar to that in mmtv-C-myc mice. In contrast the growth of these myc-dependent tumors in bigenic mice was significantly lower ($P<0.01$) than that in mmtv-C-myc mice. These data support the conclusion that while overexpression of USF-2 has minimal impact on normal mammary gland development and the initiation of mammary tumors in response to C-myc overexpression, it can slow the growth of established C-myc-dependent mammary tumors.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9070 supported this work.

ACTIVATION OF AKT/PKB INTERFERES WITH MAMMARY GLAND INVOLUTION AND PROMOTES TUMOR PROGRESSION

John N. Hutchinson,[1] Jin Jing,[2] Robert D. Cardiff,[3] Jim R. Woodgett,[2] and William J. Muller[1]

[1]McMaster University, Hamilton, ON, Canada;
[2]Ontario Cancer Institute, Toronto, ON, Canada;
[3]University of California at Davis, Davis, CA

jhutchin@mcmaster.ca

The mammary gland undergoes a regulated cycle of proliferation, differentiation and apoptosis during its development and disruption of this cycle can lead to the occurrence of many abnormalities including tumorigenesis. In conjunction with hormones and cell-substratum interactions, the growth and differentiation of mammary epithelial cells is regulated by growth factors and their receptors. Activation of these receptors leads to the recruitment of a number of cytoplasmic signaling molecules including the phosphatidylinositol 3'-OH kinase (PI3K) which plays an important role in coupling these receptors to cell survival pathways via the Akt/PKB (Protein Kinase B) serine/threonine kinase. Evidence supporting the importance of the PI3K/Akt signaling pathway in mammary tumorigenesis stems from experiments with transgenic mice bearing either polyomavirus middle T antigen (PyV mT) or activated ErbB-2 under the control of the mouse mammary tumor virus-long terminal repeat promoter. The mammary epithelial specific-expression of PyV mT results in the rapid development of multifocal metastatic mammary tumors whereas transgenic mice expressing a mutant mT de-coupled from PI3K (MTY315/322F) develop extensive mammary gland hyperplasias that are highly apoptotic which progress at long latency to become focal tumors without transgene reversion. Similarly, mice expressing activated ErbB-2 (NDL2-5) develop mammary gland hyperplasias which progress to tumors. Significantly, tumors from both of these strains overexpress ErbB-3, which possesses 7 consensus binding sites for PI3K. To directly assess the role of Akt in mammary development and tumorigenesis, we generated transgenic mice expressing constitutively active Akt (Akt-DD). Although expression of Akt-DD interferes with normal mammary gland involution, tumors were not observed in these strains. Co-expression of Akt-DD with MTY315/322F resulted in a dramatic acceleration of mammary tumorigenesis correlated with reduced apoptotic cell death. Furthermore, co-expression of Akt-DD with MTY315/322F resulted in phosphorylation of the FKHR forkhead transcription factor and translational upregulation of cyclin D1 levels.

Preliminary results indicate that co-expression of activated Akt and an activated ErbB-2 in the mammary gland increases mammary tumor formation and is associated with increased mitogenesis and cyclin D1 levels. Importantly, we did not observe wildtype metastasis levels in the bi-transgenic strains. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal but does not promote metastatic progression.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0334 and DAMD17-98-1-8123 supported this work.

HER2, HER3, AND C-SRC COOPERATIVE INTERACTIONS IN BREAST CANCER

Rumey C. Ishizawar and Sarah J. Parsons

Department of Microbiology and Cancer Center,
University of Virginia Health System

ri4n@virginia.edu

Members of the human epidermal growth factor receptor (HER) family and c-Src, an intracellular tyrosine kinase protein have been found to be co-overexpressed in a large subset (60%) of human breast cancers. This co-overexpression suggested that the two classes of tyrosine kinases may functionally interact. Our laboratory previously demonstrated that c-Src transiently interacts with the epidermal growth factor receptor (EGFR), also known as HER1, and phosphorylates it at Tyr-845 to synergistically promote mitogenesis and tumorigenesis in response to EGF. As HER2/neu has been shown to be present in up to 30% of human breast cancer cases and correlates with a poor disease prognosis, we examined whether c-Src may functionally interact with HER2/neu in a manner similar to EGFR/HER1 and if it may affect heregulin (HRG) signaling through HER3. These studies were preformed in a C3H10T1/2 murine fibroblast model system in which HER2, HER3, and c-Src are overexpressed either alone or in various combination. As shown previously by others, we found that c-Src co-associated with HER2 and HER3 in our model system. This association took place under basal conditions and was not increased by HRG stimulation, although HRG treatment did enhance association between HER2 and HER3 as described in the literature. Furthermore, the kinase activity of c-Src was not necessary for the interaction of c-Src with HER2/HER3. Results of time course and dose response studies with HRG suggested that overexpression of wild-type (wt) c-Src augments sensitivity of HER3 to respond to its activating ligand as measured by autophosphorylation. In contrast to overexpression of the receptors alone or receptors plus kinase-inactive (K-) c-Src, co-overexpression of HER2, HER3, and wt c-Src together promoted enhanced basal tyrosine phosphorylation of multiple cellular proteins and HRG induced tyrosine phophorylation of an ~85 kDa protein, that was found in a multiprotein complex with HER2, HER3 and c-Src. However, neither wt c-Src nor K- c-Src appeared to affect the ability of HER3 to promote AKT phosphorylation in response to HRG. The significance of the catalytic activity of c-Src in promoting HER2 and HER3 signaling was most evident in biological studies, measuring anchorage-independent growth by soft agar colony formation. In both the presence of serum or HRG, the expression of K- c-Src prevented HER3 from promoting colony formation. As compared to HER2/HER3 double overexpressors, HER2/HER3/wt c-Src triple overexpressors demonstrated enhanced colony formation in reduced serum conditions, which could be further elevated by the presence of HRG. Together, these data support the conclusions that c-Src physically interacts and functionally cooperates with the HER2/HER3 signaling complex to promote breast cancer cell growth. Furthermore, our findings suggests that the interaction of c-Src with HER2/HER3 differs from its interaction with EGFR. Such studies may direct future breast cancer therapy targeting.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0487 supported this work.

CHARACTERIZATION OF A LARGE MOLECULAR WEIGHT NOTCH COMPLEX IN THE NUCLEUS OF NOTCHIC-TRANSFORMED CELLS

Shawn Jeffries, David J. Robbins, and Anthony J. Capobianco

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, Cincinnati, OH 45267-0524

jeffrisk@email.uc.edu

Notch genes encode a family of transmembrane proteins that are involved in many cellular processes such as differentiation, proliferation and apoptosis. It is well established that all four Notch genes can act as oncogenes, however, the mechanism by which Notch proteins transform cells remains unknown. Previously, we reported that both nuclear localization and transcriptional activation are required for neoplastic transformation of RKE cells. Furthermore, we identified cyclin D1 as a direct transcriptional target of constitutively active Notch molecules. In an effort to understand the mechanism by which Notch functions in the nucleus we sought to determine if Notch formed stable complexes using size exclusion chromatography. Herein, we report that Nic forms distinct high-molecular weight complexes in the nuclei of transformed RKE cells. The largest complex is approximately 1.5 Mda and contains both endogenous CSL and Mastermind (Maml). Nic molecules that do not have the high-affinity binding site for CSL (RAM) retain the ability to associate with CSL in a stable complex through interactions involving Maml. However Maml does not directly bind to CSL. Furthermore, Maml can rescue ΔRAM transcriptional activity on a CSL-dependent promoter. These results indicate that deletion of the RAM domain does not equate to CSL-independent signaling. Moreover, in Sup-T1 cells Nic exists exclusively in the largest Nic-containing complex. SUP-T1 cells are derived from a T-cell leukemia that harbors the t(7;9)(q34;q34.3) translocation and constitutively express Nic. Taken together our data indicates that complex formation is likely required for neoplastic transformation by Notchic.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0202 supported this work.

TARGETING OF ERBB2 DNA WITH POLYAMIDES

Vsevolod Katritch and Ruben Abagyan

The Scripps Research Institute, Plexus Vaccine, Inc.

katritch@plexvax.com

Tyrosine kinase receptor erbB2/HER2/neu oncogene, a key component in the epidermal growth factor (EGF) signaling pathway, is amplified and upregulated in 25-30% of human breast cancers and is associated with poor clinical prognoses. Specific inhibition of the gene on the transcriptional level (antigene strategy) would have a high therapeutic potential. We suggest using a novel class of Pyrrole-Imidazole (Py-Im) containing polyamides to bind specific DNA sequences in the erbB2 promoter region to disrupt transcription.

We have applied sequence analysis tools to identify the most promising short targets within erbB2 DNA promoter sequence and performed extensive molecular modeling to design optimal polyamide molecules that bind these dsDNA targets. We found that the region around the TATAA box, previously used as an antigen target (Chiang, S.Y. et al. J Biol Chem 275, 24246-54. (2000)), has very poor specificity in the human genome. On the other hand, we discovered sequences containing 13 bp fragments with almost unique whole-genome specificity, also overlapping with one or more erbB2 activation sites. As a result of our analysis, several short fragments erbB2 promoter sequences have been chosen as optimal targets for polyamide design, including GC and CCAAT boxes, c-Myb and AP2 regulatory sites.

A fast and reliable algorithm have been developed with ICM modeling software to build 3D models of polyamides-DNA interaction, based on the known modular structure of the complexes and all-atom conformational energy minimization with ICFF potential function (Katritch, V., Totrov, M. & Abagyan, R., J. Comp. Chem. (2002)). The affinity of the DNA - polyamide binding can be predicted by this method with an accuracy of ~1.5 Kcal/mol, which significantly narrows the search for the best candidate polyamides for future in vitro and in vivo experiments, the accuracy of such modeling demonstrated in NMR experiments (Geierstanger, B., Katritch, V., et al. J. of Am. Chem. Soc Submitted(2002)). Using polyamide virtual "residue" library we have generated more than a 100 different polyamide "sequences" for each of the dsDNA binding sites, build 3D models of these molecules and selected a few best designs according to their predicted binding affinity.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9323 supported this work.

STUDYING GENETIC INTERACTIONS LEADING TO BREAST CANCER USING A SOMATIC GENE TRANSFER SYSTEM

Yi Li and Harold Varmus

Memorial Sloan-Kettering Cancer Center

liy@mskcc.org

Although many genetic lesions are implicated in breast cancer, very little is known regarding the molecular interactions that promote breast tumorigenesis. Mouse models have helped to delineate the interactions of cancer genes in vivo, however, the progress in the use of transgenic and knockout mice to study in vivo genetic interactions has been slow. This is largely attributable to the long time and high cost associated with breeding mice with multiple genetic mutations. I have utilized the TVA system which allows the examination of multiple genetic lesions without the need to create and breed individual transgenic lines. The system is based on the use of the RCAS virus (an avian leukosis virus vector of subgroup A) to deliver genes to mammalian cells or tissues that have been engineered to produce the avian viral receptor TVA and has successfully been used to evaluate oncogenic lesions in brain and ovary tumorigenesis.

I have generated transgenic mice expressing TVA in the mammary gland using the mouse mammary tumor virus (MMTV) promoter. I have determined that the TVA receptor is produced in ductal epithelial cells of the transgenic mice and that epithelial cells from the transgenic mice are susceptible to infection with RCAS vectors. I have found that mammary tumors can be rapidly induced by mammary gland transplantation of TVA+/p53-/- primary mammary cells infected, in vitro, with RCAS vectors expressing oncogenes such as K-Ras or neu. This somatic gene delivery system may be useful for dissecting genetic interactions that operate in breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9312 supported this work.

IDENTIFYING ONCOGENES THAT COOPERATE IN MOUSE MAMMARY TUMORIGENESIS

Gregory M. Shackleford and Rocío López-Diego

Departments of Pediatrics and Molecular Microbiology and Immunology, University of Southern California and Children's Hospital Los Angeles, Los Angeles, CA 90027

shacklef@hsc.usc.edu

Our goal is to isolate and characterize oncogenes that are implicated in multistep mammary tumorigenesis. Previously, we and others have established that activated *Wnt* and *Fgf* genes are strong collaborators in this process. In order to identify additional oncogenic events involved in this process, we have used mouse mammary tumor virus (MMTV) insertional mutagenesis in two bitransgenic mouse models of breast cancer carrying the following transgenes: *Wnt1* and a dominant-negative (DN) *Fgfr2* (fibroblast growth factor receptor 2); and *Wnt1* and *Fgf3*.

In the *Wnt1/Fgfr2(DN)* model, a Wnt oncogenic signal is constitutively active in the mammary gland, while the activation of cooperative FGF signaling is blocked through the overexpression of a dominant-negative (tyrosine kinase-defective) FGF receptor. In the *Wnt1/Fgf3* model, both Wnt and Fgf oncogenic signals are activated in the mammary gland. Hence, in both models, since both the Wnt and FGF pathways are either already stimulated or are blocked, we hypothesize that insertional activation of genes other than those involved in these pathways will be selected for during MMTV-induced mammary tumorigenesis. That is, there would be no growth advantage for any insertional activations of a *Wnt* or an *Fgf* gene, for example, so only genes in other (cooperative) pathways are likely to be detected as events resulting in the clonal growth of a tumor.

Infected bitransgenic females in both models develop mammary adenocarcinomas, and frequent lung metastases were also observed. Southern blot analysis of mammary tumor DNAs confirmed the presence of newly integrated MMTV proviruses in several of these tumors. We are currently using an inverse polymerase chain reaction approach to isolate viral-cellular junction fragments from these tumors. In this way, we hope to clone common gene targets for MMTV insertional activation, and subsequently study their mammary oncogenic potential.

In conclusion, two bitransgenic mouse models of breast cancer have been generated, and cohorts of females have been infected with MMTV with the goal of identifying common insertion loci that harbor activated oncogenes that cooperate with *Wnt* and/or *Fgf* genes. Newly integrated MMTV proviruses have been detected in some tumors and these are being analyzed for activated oncogenes. The identification of oncogenes that participate in multistep mammary tumorigenesis is an important and necessary first step toward the development of rational therapies directed against the protein products of such genes.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0199 supported this work.

CONDITIONAL OVEREXPRESSION OF ACTIVATED NEU IN THE MAMMARY EPITHELIUM OF TRANSGENIC MICE RESULTS IN REVERSIBLE PULMONARY METASTASES

Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Dugan KD, Innocent N, and Chodosh LA

University of Pennsylvania School of Medicine

moody@mail.med.upenn.edu

Amplification of the proto-oncogene HER2/*neu* occurs in 20-30% of primary human breast cancers and is associated with aggressive tumor behavior and poor prognosis. Consistent with this, transgenic mice constitutively overexpressing activated *neu* in the mammary epithelium under the control of the mouse mammary tumor virus (MMTV) long terminal repeat develop invasive mammary adenocarcinomas with short latency. To determine whether mammary tumors initiated by *neu* remain dependent on *neu* overexpression for continued growth, we used the tetracycline regulatory system to inducibly express activated *neu* in the mammary epithelium of transgenic mice. These mice rapidly develop multiple invasive mammary adenocarcinomas in all mammary glands in a doxycycline-dependent manner. Furthermore, a large percentage of animals maintained on doxycycline after initial tumor detection eventually develop pulmonary metastases. Notably, nearly all *neu*-induced primary tumors were found to regress to a clinically undetectable size following doxycycline withdrawal. Moreover, despite the acquisition of genetic changes that render tumor cells able to metastasize, the majority of lung metastases arising from *neu*-induced mammary tumors were also found to regress fully following doxycycline withdrawal. These findings suggest that the vast majority of cells in *neu*-induced tumors and metastatic lesions remain dependent on the initiating oncogenic stimulus for maintenance of the transformed state. Given that metastatic breast cancers are rarely cured, even with the use of combinatorial therapies, these findings appear to contradict clinical experience. In fact, despite the dramatic clinical response that we have observed following abrogation of the initiating oncogenic stimulus, the majority of deinduced transgenic mice eventually develop tumor recurrences in the absence of doxycycline. This observation parallels the natural history of human breast cancers treated by targeting a single specific oncogenic pathway.

Our findings highlight two important features that warrant consideration in the development of new treatments for human cancers: first, that the majority of tumor cells remain dependent upon a single oncogenic mutation even in advanced stages of disease, thereby rendering these cells susceptible to targeting of the oncogenic pathway altered by that mutation; and, second, that targeting a single pathway is unlikely to effect a cure. Our model provides a system in which the secondary pathways that lead to tumor recurrences can be analyzed, as can the nature of residual disease harbored by animals in which tumors have fully regressed, with the ultimate goal of designing multi-target therapeutic strategies that achieve tumor cell eradication.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0401 supported this work.

FUNCTIONAL SITES OF THE ERBB-2 RECEPTOR AND ITS ACTIVATOR, HEREGULIN

Joanna E. Mroczkowska-Jasinska, Kelly M. Quinn, and Ruth Lupu

Lawrence Berkeley National Laboratory,
Berkeley, CA 94720

jemroczkowska@lbl.gov

Over expression of the erbB-2 receptor occurs in up to 30% of cases of invasive breast carcinomas and correlates with aggressive diseases and poor prognosis for therapy and poor overall survival. The growth factor heregulin (HRG) binds to erbB-3 or erbB-4 receptors, promoting heterodimerization with erbB-2, and induces autophosphorylation and activation of erbB-2 signaling. It is generally accepted that heregulin and erbB-2 do not interact directly. Depending on its concentration HRG can either inhibit or stimulate cell proliferation in cell lines that overexpress erbB-2. These suggest a possible weak interaction between HRG and erbB-2. Solution structure of HRG as well as other data supports the existence of a low-affinity binding site for erbB-2 within the EGF-like domain of HRG. Moreover we have shown that a synthetic peptide, derived from sequence at the erbB-2 extracellular domain, was capable of specifically block HRG-induction of erbB-2 tyrosine phosphorylation. Therefore it is possible that this region constitutes a critical region of the erbB-2 receptor responsible for HRG induction of erbB-2 heterodimerization and activation. To determine the putative sites involved in the interactions between HRG and the erbB-2 receptor, HRG and erbB-2 specific deletion mutants were generated. Eight deletions were generated within the extracellular domain of erbB-2 and transduced into Ba/F3 cells to determine the mechanism of interaction between erbB-2 and heregulin. To determine whether the deletions on erbB-2 contained a site controlling HRG induced heterodimerization and tyrosine phosphorylation, deletion of the erbB-2 receptor were introduced into BaF3 cells. These cells were previously shown to express erbB-3 and when transfected with the erbB-2 were no longer dependent upon IL-3 and were dependent upon HRG. Our preliminary data suggests that erbB-2 deletion proteins did not contain the functional sites for activation, and lose the ability of autophosphorylation. To define the HRG site involved in any interactions with erbB-2, five different point mutations within the EGF-like domain of the HRG beta 2 gene were introduced. Purified recombinant proteins containing introduced mutations are currently used in the experiments to determine the mechanism of interaction between erbB-2 or HRG. Our studies will shed light into the specific erbB-2 functional site(s). The information will provide evidence for the generation of specific chemotherapeutic drugs to target these proteins functional site(s).

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9249 supported this work.

IDENTIFICATION OF c-MYC ONCOPROTEIN-INTERACTING PARTNERS USING THE NOVEL REPRESSED-TRANSACTIVATOR ASSAY (RTA)

Cynthia S. W. Ho,[1] Martin Hirst, Ph.D.,[2] Ivan Sadowski, Ph.D.,[2] and Linda Z. Penn, Ph.D.[1]

[1]Cellular and Molecular Biology, Ontario Cancer Institute, ON, Canada; [2]Faculty of Medicine, University of British Columbia, BC, Canada

hoc@uhnres.utoronto.ca

Deregulation of c-myc oncogene contributes to a wide variety and large number of human malignancies, including breast carcinoma. Myc is a nuclear phosphoprotein that functions as a regulator of gene transcription to drive numerous biological activities including transformation. Experimental evidence strongly suggests that protein interactions occurring at the N-terminal domain (NTD) of Myc are key to the malignant potentiation of Myc protein function and isolation of these cellular partners will allow us to design inhibitors that would directly target Myc-mediated transformation.

We have established a novel yeast two-hybrid screen, termed the Repressed Transactivator Assay (RTA), to identify and characterize Myc NTD protein interactions. Myc binding studies have traditionally relied upon labour intensive biochemical techniques or yeast two-hybrid screening with a severely truncated Myc bait. These methods lack either the high throughput capacity of a functional screen or are deficient in preserving the native Myc NTD structure and activity, respectively. As an *in vivo* high throughput functional screen, the RTA overcomes these limitations and extends the current technology. Using a panel of control interactions we show that the RTA can distinguish between positive and negative Myc interactions in a sensitive and specific manner. The development of the RTA is particularly exciting because it enables us, for the first time, to use the entire functional Myc NTD as bait in a two-hybrid system. This assay has allowed us to map quickly the binding sites of known interactions and to efficiently screen cDNA libraries to identify novel Myc binding partners.

To identify novel interactors, we have used the Myc NTD bait to screen a cDNA library derived from a human breast cancer cell line, MCF-7. In healthy breast epithelium, Myc induction is associated with the proliferative phases of development where growth occurs in a tightly controlled manner. By contrast, MCF-7 cells are transformed and contain an activated level of Myc expression. Myc is important for the growth of this cell line as Myc levels become further induced in response to estrogen stimulation and introduction of anti-sense Myc inhibits MCF-7 cell proliferation. Thus, it is reasonable to propose that protein partners important for Myc's potent ability to promote carcinogenesis may be found in this context. To date, we have established the RTA system and successfully completed a library screen using Myc NTD as bait. We are currently characterizing the candidate clones for their ability to interact with Myc in vitro and in vivo. As well, studies are underway to determine the functional consequences of these interactions on Myc-induced transformation and apoptosis. As the molecular interplay between Myc and its interacting proteins becomes better understood, the ability to develop cancer therapies that specifically target Myc function in transformation or that enhance Myc-induced cell death, will be possible.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0434 supported this work.

BLOCKING HER-2/HER-3 FUNCTION USING A DOMINANT NEGATIVE FORM OF HER-3 IN BREAST CANCER CELLS WITH HER-2 GENE AMPLIFICATION

Tracy G. Ram, Ph.D.

School of Biological Sciences, Washington State University, Pullman, WA 99164-4236

tram@mail.wsu.edu

Cooperative interactions between HER-2 (neu/erbB-2) and HER-3 (erbB-3) constitutively activate PI 3-kinase and MAP-kinase during the autonomous growth of breast cancer cells with HER-2 gene amplification. Therefore, we constructed a bicistronic retroviral expression vector containing a dominant negative form of HER-3 (DN3) in order to block HER-2/HER-3 heterodimer function in breast cancer cells. 21MT-1 cells, which have HER-2 amplification and constitutive activation of HER-2/HER-3, express EGFR and are highly responsive to the mitogenic effects of EGF as well as heregulin (HRG), the ligand for HER-3, in serum-free culture. The ectopic expression of DN3 in 21MT-1 cells was able to effectively block both the constitutive and HRG-stimulated activation of HER-2/HER-3 in culture. While EGFR activation and cell proliferation stimulated by EGF was not apparently affected by DN3, growth factor-independent (i.e. autonomous) proliferation, HRG-stimulated proliferation and anchorage-independent growth in agarose were potently inhibited by DN3. However, 21MT-1 cells were not sufficiently tumorigenic in immunodeficient mice for in vivo analysis. Therefore, we infected additional breast cancer cell lines containing HER-2 amplification with DN3, such as BT-474 cells, which were tumorigenic in scid mice and showed high-level constitutive activation of HER-2/HER-3. In contrast to cells infected with control vector, many BT-474, MDA-MB-453 or SK-BR-3 cell colonies infected with DN3 grew very slowly or died shortly after selection on G418. Small slow growing and dying colonies expressed high levels of DN3, while larger fast growing colonies showed only lower levels of DN3, indicating that we were selecting for cells expressing only lower levels of DN3 and that constitutive HER-2/HER-3 activation was required for the continuous proliferation of these cell lines grown with 10% serum in culture. Therefore, work is underway using a tetracycline-inducible DN3 expression vector for assessing the effectiveness and stoichiometry of DN3 inhibition of HER-2/HER-3 function in various breast cancer cell lines with HER-2 amplification. These studies suggest an important role for constitutive HER-2/HER-3 activation in breast cancer as well as a new strategy for intervention.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0490 supported this work.

C-REL OR ACTIVATION OF NF-κB BY PROTEIN KINASE CK2 PROMOTES BREAST CANCER

R. Romieu-Mourez, D. W. Kim, S. Shin, E. Landesman-Bollag, D. C. Seldin, and G. E. Sonenshein

Boston University School of Medicine

romieu@biochem.bumc.bu.edu

Nuclear Factor-kappaB (NF-κB) transcription factors regulate genes that control cell proliferation, survival, and transformation. Breast cancers have been characterized by aberrant activation of c-Rel, p65/RelA, p50, and p52 NF-κB/Rel subunits. Protein kinase CK2 is upregulated in many human breast cancer specimens and cell lines, including Hs578T human breast cancer cells, and NF639 mammary tumor cells derived from an MMTV-Her-2/neu transgenic mouse. Previously, stability of IκB and transcriptional activity of NF-κB have been shown to be regulated by phosphorylation at CK2-specific sites. Here, we have tested the hypothesis that CK2 can directly induce basal NF-κB activity. Inhibition of CK2 activity was directed by stable expression of kinase-inactive forms of CK2 catalytic subunits in Hs578T and NF639 breast cancer cell lines downregulated the level of NF-κB, and strongly increased IκB-α half-life. Importantly, cells expressing kinase-inactive CK2 displayed enhanced susceptibility to TNF-mediated cell death and reduced ability to grow in soft agar. Overexpression of CK2 by retroviral gene delivery led to induction of classical NF-κB (p50/RelA), and increased IκB-α turnover in NIH 3T3 fibroblast cells. These observations suggest that CK2 upregulation plays a direct role in an intracellular signaling pathway that leads to the elevated NF-κB levels seen in primary human mammary tumors, and therefore represents a potential therapeutic target in the treatment of patients with breast cancer.

To directly evaluate the contribution of NF-κB overexpression to mammary tumorigenesis, we targeted c-Rel to the mammary gland, under control of the MMTV-LTR promoter. Four transgenic mouse founder lines were isolated, which displayed c-Rel transgene expression in the mammary glands induced by pregnancy. Thirty-five percent of the female MMTV-c-Rel transgenic mice that lived to 2 years of age developed mammary tumors at a median age of 19.7 months. Tumors were associated with NF-κB activation, and upregulation of NF-κB target genes, e.g., cyclin D1 and Bcl-x_L. Thus, c-Rel overexpression in mice is sufficient to induce or breast tumorigenesis. This is the first report demonstrating the *in vivo* transforming ability of c-Rel, the cellular homologue of highly tumorigenic v-Rel oncogene. Overall these studies indicate a critical role for NF-κB in breast tumorigenesis.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0158 supported this work.

TRANSFORMATION OF HUMAN CELLS IN THE ABSENCE OF TELOMERASE ACTIVATION

Yvette R. Seger,[1] Roberta Maestro,[2] Sara Piccin,[2] Crucifissa Lo Cunsolo,[3] Marta Garcìa-Cao,[4] Claudio Doglioni,[3] Marìa A. Blasco,[4] and Gregory J. Hannon[1]

[1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; [2]CRO National Cancer Institute, Aviano, Italy 33081; [3]Department of Histopathology, Belluno City Hospital, Belluno, Italy 32100; [4]National Centre of Biotechnology (CSIC), Madrid, Spain 28049

seger@cshl.org

Normal cells become transformed into tumor cells via cooperating genetic and epigenetic events, which result in the activation of oncogenes and the inactivation of tumor suppressors. In primary rodent fibroblasts, this process is well-defined and usually requires two genetic hits. However, these rodent transformation models do not perfectly recapitulate the transformation process in humans as it is impossible to transform normal human cells into tumor cells using the same oncogene combinations that are effective in rodent cells. One obvious difference between the rodent and human models is the requirement for telomerase, the enzyme responsible for maintaining telomeres. In rodent cells, transformation is independent of exogenous telomerase induction in part because of promiscuous telomerase expression and in part because of constitutively long telomeres. The potential importance of telomerase in human transformation processes has been supported by the fact that a majority of human tumors have an induction of telomerase expression. In addition, previous reports have indicated that direct expression of the telomerase catalytic subunit hTERT is a vital component of the human cell transformation equation.

We have transformed primary human foreskin fibroblasts (BJ) without direct introduction of hTERT or a gene previously shown to be capable of activating telomerase. BJ cells expressing the gene combination of adenovirus E1A, MDM2, and Ha-RasV12 are capable of both soft agar colony formation and tumor formation in nude mice. These cells are telomerase negative upon injection into nude mice, and the resultant tumors are also negative for telomerase activity. As a result, we see continuous telomere shortening and chromosomal abnormalities similar as to what had been seen in telomerase-null mice.

Substitution of wild-type E1A with deletion mutants has allowed us to further characterize the functional interactions that may be important for both transformation processes and the ability to evade normal mortality controls in human fibroblasts. In addition, substitution of MDM2 with either Bcl-2 or a dominant-negative form of p53(175H) indicates that MDM2 is functioning in this transformation equation through the p53 pathway. In addition, several primary human fibroblast cell lines have now been transformed utilizing this oncogene combination.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0210 supported this work.

A NEW ANIMAL MODEL FOR MAMMARY ONCOGENE DISCOVERY

Gregory M. Shackleford and Roció López-Diego

Departments of Pediatrics and Molecular Microbiology and Immunology, University of Southern California and Children's Hospital Los Angeles, Los Angeles, CA 90027

shacklef@hsc.usc.edu

Mammary oncogenes encode the proteins that are causally related to the genesis of breast cancer. An important model for mammary oncogene discovery has been the "retroviral tagging" of such genes by mouse mammary tumor virus (MMTV), which occurs when MMTV integrates a DNA copy of its genome into a mouse mammary cell chromosome during infection. Nearby genes are often upregulated or directly mutated by such integrations, which can result in tumor formation if the activated gene is involved in the regulation of cell growth. Since the strain of mouse has been shown to be important in determining which genes are found using this strategy, we are using the rat as a new host for experimental infection with MMTV, so that a potentially new repertoire of mammary oncogenes may be detected.

We used three strains of rats that are known to develop mammary tumors under appropriate conditions: Sprague Dawley, Fischer 344 and Wistar. To determine if MMTV could infect rat tissues, we (i) injected MMTV subcutaneously in rats and examined the draining lymph nodes for enlargement (a sign of MMTV infection), (ii) examined the DNAs of these lymph nodes of evidence of MMTV proviral DNA by PCR, and (iii) introduced MMTV directly into rat mammary gland ducts and examined DNAs of these glands several days later for evidence of MMTV proviral DNA.

We found that after subcutaneous injection of MMTV, the draining lymph nodes were dramatically enlarged six days after injection, suggesting that MMTV infected the lymphoid cells and that the MMTV-encoded superantigen functions in the rat. Genomic DNA was isolated from these lymph nodes and found to be positive for MMTV by PCR analysis, thus confirming that MMTV could indeed infect lymphoid cells of the rat. This was significant, since infection of lymphoid cells is a requisite step in the natural (i.e., milk route) infection process in mice. Direct injection of MMTV into the mammary ductal system, together with hormone treatments to stimulate mammary cell proliferation, resulted in infection of mammary gland cells as shown by MMTV-positive PCR.

We conclude that the lymphoid and mammary gland cells of rats are infectable by MMTV. We have infected all three strains of rats with MMTV and will examine the DNAs of any tumors that arise for the insertion of new MMTV DNAs and for activated oncogenes. The identification of mammary oncogenes is necessary to identify targets for new therapeutics.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0200 supported this work.

DUAL-SPECIFICITY HER-2/NEU ANTISENSE

M. J. Leibowitz, J. Perlman, and S. Stein

University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Cancer Institute of New Jersey, Center for Advanced Biotechnology and Medicine

leibowit@umdnj.edu

The HER-2/neu (erbB2) gene product is over-expressed in about 1/3 of breast cancers, and this correlates with poor prognosis. The Herceptin monoclonal antibody, directed against this protein, is therapeutically useful, enhancing the efficacy of some chemotherapeutic agents. Therefore, we are studying a new antisense approach to down-regulation expression of erbB2. The 5' untranslated region (UTR) of mRNA is often the most effective target for antisense DNA, which acts by sensitizing mRNA to digestion by RNase H. High affinity specific binding to a target on mRNA requires an antisense DNA of at least 15 nucleotides. We have found that antisense DNA hexamers targeted to unpaired regions of the 5' UTR of erbB2 mRNA are capable of stimulating RNase H cleavage at their sites of binding, although their efficiency is low and they lack specificity due to their small size. By coupling a 2'-O-methyl RNA hexamer via linkers of various lengths, to a phosphorothioate DNA hexamer, both binding to such unpaired regions of the 5' UTR, we have created dimeric antisense compounds that specifically sensitize the RNA at the DNA hexamer binding site, with specificity conferred both by the sequence and spatial arrangement of the 2 hexamer molecules, as indicated by dependence of antisense activity on linker size. These dimeric constructs have increased affinity for their target relative to individual hexamers. We have also tested the effect of these dimeric antisense compounds on translation of mRNA constructs with the 5' UTR of erbB2 linked to the luciferase reporter gene. We found that the dimeric antisense constructs that most sensitized the 5' UTR to RNase H also most inhibited translation of this mRNA in wheat-germ and rabbit reticulocyte cell-free systems; these constructs were more potent that an 15-mer phosphorothioate targeting a site immediately 5' to the initiating AUG. However, we have not been successful in introducing the dimeric antisense constructs into cultured breast cancer cells to test their effect on erbB2 expression. We are currently testing new methods of delivering these novel antisense agents into cells.

Original work supported by the U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7288.

THE ESSENTIAL ROLE OF AKT-1/PROTEIN KINASE B IN PTEN-CONTROLLED TUMORIGENESIS

Bangyan Stiles, Valarie Gilman, Natalya Khnzenzon, Ralph Lesche, Annie Li, Qiao Rong, and Hong Wu[1]

Department of Molecular and Medical Pharmacology,
University of California, Los Angeles, School of Medicine,
Los Angeles, CA 90095-1735

bstiles@mednet.ucla.edu

PTEN is mutated at high frequency in many primary human cancers and several familial cancer predisposition disorders. Activation of AKT is a common event in tumors in which PTEN gene was deleted or mutated. We showed previously that deletion of murine Pten gene in embryonic stem cells (ES) led to increased PIP3 accumulation, enhanced entry into S phase, and better cell survival. Since PIP3 controls multiple signaling molecules, it was not clear to what degree that the observed phenotypes were due to deregulated AKT activity. In this study, we mutated Akt-1 in Pten-/- ES cells to direct assess the role of AKT-1 in PTEN controlled cellular processes, such as cell proliferation, cell survival, and tumorigenesis in nude mice. We demonstrated that AKT-1 is a major downstream effector of PTEN in ES cells and that activation of AKT-1 is responsible for both the cell survival and cell proliferation phenotypes observed in Pten-/- ES cells. Deletion of Akt-1 also reverses the aggressive growth of Pten-/- ES cells in vivo, suggesting that AKT-1 plays an essential role in PTEN controlled tumorigenesis.

The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0182 supported this work.

BAG-FAMILY PROTEINS IN BREAST CANCER

Takayama, S., and Homma, S.

The Burnham Institute

stakayama@burnham.org

BAG-family proteins regulate diverse cellular functions, including cell survival, cell proliferation, and cell motility. Over-expression of BAG1 has been demonstrated in breast cancer.

BAG-family proteins contain a conserved domain that allows them to bind 70-kD heat shock (Hsp70) family molecular chaperones and regulate their activity (Takayama, et al EMBO J 16: 4887, 1997). BAG-family proteins are evolutionaly conserved with six mammalian, one Drosophila, two C.elegans, two S.pombe, one S.cerevisiae and multiple Arabidopsis taliana homologues identified thus far (Takayama et al J Biol Chem 274: 781, 1999). Structural analysis of the Hsc70-binding BAG domain of BAG1 has revealed an anti-parallel two helix bundle, proceeded by an additional long alpha-helix. Site-directed mutagenesis has confirmed that the polar surfaces of the alpha-helices in the BAG domain are directly involved in chaperone binding, which has been confirmed by NMR experiments. Similarly, an 80 amino acid region (229-308) of Hsc70 has been determined to represent a minimal domain sufficient for binding the BAG domain. The conserved BAG domains of BAG1 family protein from plant and yeast also bind the Hsc70 ATPase domain. In addition to the Hsp70-binding domain, BAG-family proteins also contain a diversity of additional domains, which allow them to interact with specific target proteins or which target them to specific locations within cells.

We generated polyclonal antisera against recombinant BAG2 and BAG3 protein. Western blot analysis of the NIH panel of 60 cancer cell lines revealed overexpression of BAG3 in breast cancer cell lines, but not BAG2. The BAG3 protein contains a WW domain and a proline-rich region with SH3-binding motifs, suggesting that it may interact with proteins relevant to submembranous signal transduction, recruiting Hsp70/Hsc70 to signaling complexes and altering cell responses. Based on preliminary evidence that BAG3 may be over-expressed in cancers, we speculate that this protein represents a novel proto-oncogene that may affect cell transformation, anchorage-independent growth, or other processes relevant to the malignant phenotype. Recently, we identified interacting proteins and their role in BAG3-dependent phenotypes is under investigation.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9094 supported this work.

RIBOZYME TARGETING THE NOVEL FUSION JUNCTION OF EGFRVIII IN BREAST CANCER

Xunyi Luo, Xiaoqi Gong, and Careen K. Tang, Ph.D.

Lombardi Cancer Center, Department of Oncology,
Georgetown University Medical Center, Washington, DC

tangc@georgetown.edu

EGFRvIII is a tumor specific, ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has been detected in many human malignancies including breast cancer. In our previous study, we demonstrated that expression of EGFRvIII in MCF-7 cells produced a constitutively activated EGFRvIII receptor. These MCF-7/EGFRvIII transfectants exhibited approximately a three-fold increase in proliferation and significantly enhanced tumorigenicity of MCF-7 cells in athymic nude mice. More than 60% of invasive human breast carcinomas express EGFRvIII; no detectable level of EGFRvIII was observed in normal breast tissues. These unique features of the EGFRvIII make it an excellent target for biologically based therapies.

We have designed and generated a tumor specific ribozyme targeted to this novel EGFRvIII molecule. This specific EGFRvIII ribozyme is able to effectively cleave EGFRvIII mRNA under physiological conditions in a cell-free system, but does not cleave wild-type EGFR and other EGF-family receptors. While expressing this EGFRvIII-ribozyme in breast cancer cells, EGFRvIII-ribozyme is capable of down-regulating endogenous EGFRvIII expression at the mRNA and protein levels. Inhibition of proliferation was observed in EGFRvIII-ribozyme transfectants. In addition, down-regulation of EGFRvIII in breast cancer cells reduced tumoriginicity in athymic nude mice. Furthermore, this ribozyme has no effects on EGF-family receptor expression and proliferation in breast cancer cells, which do not express EGFRvIII but express wild-type EGFR and other EGF-family receptors. These results suggest that we have generated a tumor-specific biologically functional ribozyme and further demonstrate that EGFRvIII play a significant role in breast cancer cell proliferation.

The ultimate goal of this approach is to provide a potential treatment for breast cancer by specifically targeting this receptor.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9206 supported this work.

THE RHOC TRANSGENIC MOUSE AS A REALISTIC MODEL OF INFLAMMATORY BREAST CANCER

Melinda M. DiVito, LiWei Bao, M.D., Min Lin, M.D., Sofia D. Merajver, M.D., Ph.D., and Kenneth L. van Golen, Ph.D.

Department of Internal Medicine,
Division of Hematology/Oncology,
The University of Michigan Comprehensive Cancer Center

kgolen@umich.edu

Inflammatory breast cancer (IBC) is a phenotypically distinct form of locally advanced breast cancer characterized by a rapid onset of disease and invasive properties. Because of its aggressive phenotype, the average 5-year disease-free survival rate for IBC is less than 45%, the worst of all breast cancers. Clinically IBC is well-defined, however little was known about the genetic mechanisms underlying the disease. Our laboratory identified two genes whose expression is significantly altered in IBC tumors. One gene, RhoC GTPase, a putative oncogene responsible for cellular motility, is overexpressed and active in IBC tumors. Subsequently, we demonstrated that RhoC is a transforming oncogene for human mammary epithelial (HME) cells. Stable HME-RhoC transfectants have the ability to grow under anchorage independent conditions, are motile and invasive, produce angiogenic factors and form tumors in nude mice. We therefore hypothesize that expression of active RhoC is key in the formation of aggressive IBC.

To date only a few models exist to study IBC, including the MARY-X tumor xenograft and a few cell lines. However, these models only examine late stage tumors and do not address IBC formation. We therefore proposed to produce a RhoC transgenic mouse. We constructed 2 different expression systems for producing RhoC transgenic mice. The first system uses a tetracycline (tet)-inducible cytomegalovirus (CMV) promoter, resulting in controlled expression of wild-type RhoC. This model is useful for studying the interaction of RhoC with other oncogenes such as Ras or Her2/neu. The second system utilizes a mouse mammary tumor virus (MMTV) promoter driving expression of a constitutively active (G14V) RhoC. This system drives RhoC expression specifically in the mammary tissue of the mice. Transgenic animals have recently been produced and experiments are underway to determine the effect of inducible and constitutively active RhoC expression on mammary tumor development. RhoC expressing mice appear healthy and normal. The animals are currently being monitored for spontaneous tumor growth. Information from these experiments will provide information on how IBC tumors develop. The RhoC transgenic mouse will also provide a novel model to test new therapies.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0637 supported this work.

DEFINING THE EXPLICIT CONTRIBUTION OF SIGNALING PROTEINS TO GROWTH AND SURVIVAL OF BREAST TUMOR CELLS

Latha Shivakumar, Yu-Chen Chien, Jackie Thomas, and Michael A. White

University of Texas Southwestern Medical Center

michael.white@utsouthwestern.edu

A concerted effort of the cancer research community is well underway to identify key molecular events responsible for the initiation and progression of neoplastic cell transformation. The efforts of hundreds of laboratories have identified a wide array of bonafide and candidate oncogenes and tumor suppressors. In addition, many growth promoting genetic pathways and signal transduction cascades have been characterized and are found to be corrupted in tumor cells. Many of the observations implicating specific proteins and pathways in the initiation and progression of cancer are based on correlative and/or circumstantial evidence. Given the limited capacity of current experimental systems, only a small percentage of candidate oncogenes and tumor suppressors have been validated with genetic models. Recently, high efficiency, high specificity, gene silencing has been demonstrated in human cell culture using short interfering RNA duplex (siRNA)-mediated RNA interference (RNAi). This innovative technology facilitates the unprecedented opportunity for broad-spectrum analysis of protein function in human cancer in the context of functional knock-outs. We have shown that RNAi is highly effective in human breast tumor cell lines and in primary human breast epithelial cells. We have 'knocked-down" the expression of receptors, scaffolds, small GTPases, kinases, and transcription factors. We have found that these knock-downs behave as loss-of-function mutants and can be used to assess the explicit contribution of proteins to cell regulatory events. Here we describe the application of RNAi technology to the identification and characterization of signal transduction cascades critical for maintenance and/or initiation of breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0441 supported this work.

OVEREXPRESSION OF THE INTEGRIN-LINKED KINASE IN THE MAMMARY EPITHELIUM RESULTS IN MAMMARY GLAND HYPERPLASIA AND TUMORIGENESIS IN TRANSGENIC MICE

D. E. White, R. D. Cardiff, S. Dedhar, and W. J. Muller

McMaster University

dwhite@mcmaster.ca

The integrin-linked kinase (ILK) is a cytoplasmic effector of integrin receptors, involved in the regulation of integrin binding properties as well as the activation of cell survival and proliferation pathways, including those involving MAP kinase, PKB/Akt and GSK-3b. Overexpression of ILK in cultured intestinal and mammary epithelial cells has been previously shown to induce changes characteristic of oncogenic transformation, and ILK protein levels have been found to be elevated in various human cancers exhibiting invasive phenotypes. In order to determine if ILK overexpression can directly contribute to mammary tumorigenesis in vivo, we generated transgenic mice overexpressing ILK in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). By the age of 6 months, female MMTV/ILK mice developed a hyperplastic mammary phenotype, accompanied by the constitutive phosphorylation of PKB/Akt, GSK-3b and MAP kinase. Focal mammary tumors, showing evidence of an epithelial-to-mesenchymal transition, subsequently appeared in 34% of the animals after 1 year, with an average age of onset at 18 months. These results demonstrate that the overexpression of ILK can transform the mammary epithelium in vivo, and suggest that the elevated levels of ILK in human tumors is physiologically relevant to tumor formation and progression. ILK may therefore provide a novel therapeutic target or prognostic indicator in the treatment of human breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9284 and DAMD17-99-1-9286 supported this work.

EXPRESSION SCREENING FOR INVASION-INDUCING PROTEINS

Gwendolyn M. Mahon and Ian P. Whitehead

University of Medicine and Dentistry of New Jersey,
New Jersey Medical School

whiteip@umdnj.edu

Studies aimed at identifying genes whose deregulated expression either contribute to, or accompany, the development of breast cancer have been relatively unsuccessful. This can be largely attributed to the technical difficulties associated with the transfer of large libraries of cDNAs into human breast epithelial cells. Thus, novel oncogenes are still identified using traditional, fibroblast-based transformation assays, even though most prove to be biologically inert when expressed in epithelial cells. Since most human breast tumors are of epithelial origin, it is imperative that these technical limitations be addressed. Here we describe the development of a novel, epithelial-based expression cloning system that can be used to identify genetic sequences whose deregulated expression contribute to breast tumor progression. The general approach to cloning invasion-promoting cDNAs is to make expressible cDNA libraries from highly invasive human breast tumor cell lines, and to transfer these libraries to human tumor cells that normally do not exhibit invasive properties. Cells that acquire an invasive phenotype as a result of the expression of a transferred cDNA are selected out of the population using a transwell-based selection protocol. cDNAs are then recovered by PCR, and sequenced to determine their identity. cDNA library transfer is achieved through the use of retroviral vectors and transient packaging systems, which enables the transfer of complex cDNA libraries and their expression at consistently high levels. Retroviral vectors also permit the use of human breast epithelial cells as recipients, which is critical for identifying proteins that participate in signal transduction pathways that are relevant to epithelial cells. As a preliminary test of this system, we screened an expression library derived from the MBA-MB-231 cell line. MCF-7-EcoR cells were stably infected with retroviral particles derived from the library. Five cell populations (50,000 cells each) were screened for invasive clones. Following three rounds of library enrichment, DNA was isolated and discrete proviral inserts were identified by PCR. Sequence analysis and transforming properties of these inserts will be discussed.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0365 supported this work.

OVEREXPRESSION OF THE PROLYLISOMERASE PIN1 IN BREAST CANCER LEADS TO CYCLIN D1 ACCUMULATION

Wulf G., Ryo A., Lee S., and Lu K. P.

Beth Israel Deaconess Medical Center/
Harvard Medical School

gwulf@caregroup.harvard.edu

Phosphorylation on serines or threonines preceding proline (Ser/Thr-Pro) is a key mechanism for the control of cell proliferation and transformation. The phosphorylated Ser/Thr-Pro moiety exists in two distinct cis and trans conformations and their conversion is specifically catalyzed by the unique prolyl isomerase Pin1. Pin1 binds and regulates the function of a defined subset of phosphoproteins and is critical for cell growth. Inhibition of Pin1 induces cells to enter premature mitosis and apoptosis and also contributes to neuronal death in Alzheimer's disease. However, little is known about role of Pin1 in cancer or in modulating transcription factor activity. Here we report that Pin1 is strikingly overexpressed in most human breast cancers, and that its levels correlate with cyclin D1 levels in tumors. In cell lines derived from breast cancer, overexpression of Pin1 increases levels of cellular cyclin D1 protein and mRNA, and activates its promoter through the AP-1 site. Furthermore, Pin1 binds c-Jun that is phosphorylated on Ser63/73-Pro motifs by activated JNK or oncogenic Ras. Moreover, Pin1 cooperates with either activated Ras or JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter; these effects depend on both the isomerase activity of Pin1 and phosphorylation of c-Jun on Ser63/73. Thus, Pin1 is up-regulated in human tumors and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. Given the crucial roles of Ras signaling and cyclin D1 overexpression in oncogenesis, our results suggest that overexpression of Pin1 may promote tumor growth.

The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9081 supported this work.